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出版情報：Journal of Neuroscience. 22 (11), pp.4412-4417, 2002-06-01. Society for Neuroscience
バージョン：

Reduction of KCC2 Expression and $GABA_A$ Receptor-Mediated Excitation after *In Vivo* Axonal Injury

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After axotomy, application of muscimol, a $GABA_A$ receptor agonist, induced an increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) in dorsal motor neurons of the vagus (DMV neurons). Elevation of $[Ca^{2+}]_i$ by muscimol was blocked by bicuculline, tetrodotoxin, and Ni^{2+}. In axotomized DMV neurons measured with gramicidin perforated-patch recordings, reversal potentials of the $GABA_A$ receptor-mediated response, presumably equal to the equilibrium potential of Cl^-, were more depolarized than that in intact neurons. Thus, $GABA_A$ receptor-mediated excitation is suggested to be attributable to Cl^- efflux out of the cell because of increased intracellular Cl^- concentration ($[Cl^-]_i$) in axotomized neurons. Regulation of $[Cl^-]_i$ in both control and injured neurons was disturbed by furosemide and bumetanide and by manipulating cation balance across the membrane, suggesting that functional alteration of furosemide-sensitive cation–Cl^- cotransporters is responsible for the increase of $[Cl^-]_i$ after axotomy. *In situ* hybridization revealed that neuron-specific K^+-Cl^- cotransporter (KCC2) mRNA was significantly reduced in the DMV after axotomy compared with that in control neurons. Similar expression of Na^+, K^+-Cl^- cotransporter mRNA was observed between axotomized and control DMV neurons. Thus, axotomy led to disruption of $[Cl^-]_i$ regulation attributable to a decrease of KCC2 expression, elevation of intracellular Cl^-, and an excitatory response to GABA. A switch of GABA action from inhibitory to excitatory might be a mechanism contributing to excitotoxicity in injured neurons.

Key words: axotomy; GABA; excitation; motoneuron; NKCC1; Cl^-; Ca^{2+}

Many factors contribute to the diverse consequences of neuronal injury. Recently, attention has focused on elucidating the mechanisms contributing to the functional changes observed in neurons after injury and *in vivo*. However, most of the information regarding functional changes in injured neurons comes from peripheral neurons. In *Aplysia* sensory neurons, *in vivo* axotomy induced neuronal hyperexcitability (Gunstream et al., 1995). The reappearance of immature characteristics has been reported in injured neurons of vertebrates, such as α3, β4, and α5 ACh receptor subunits in chick ciliary ganglion (Levey and Jacob, 1996) and type III Na^+ channels in spinal sensory neurons (Waxman et al., 1994). In contrast to peripheral neurons, less is known about the responses of mammalian central neurons to *in vivo* axon injury. Recent studies demonstrate that the expression and function of glutamate receptors are altered in injured CNS neurons. Reduction in the sensitivity of NMDA receptors to extracellular Mg^{2+} (Furukawa et al., 2000) and of NR2A subunit expression have been demonstrated in vagal motoneurons after *in vivo* axotomy (Nabekura et al., 2002). Glutamate receptor subtype 1 (GluR1) (Ginsberg et al., 1996) and GluR2/3 (Alvarez et al., 2000) are downregulated in cortical neurons with axotomy. Traumatic injury has been shown to decrease Na^+-K^+ ATPase activity, resulting in membrane depolarization (Ross and Soltesz, 2000). However, alterations of neuronal responses to inhibitory transmitters after injury remain unclear.

GABA and glycine are major inhibitory transmitters in the brain and are altered in various physiological and pathophysiological conditions. In immature neurons, GABA and glycine have excitatory actions (Cherubini et al., 1990; Kakazu et al., 1999; Ehrlich et al., 1999) because of a high intracellular Cl^- concentration ($[Cl^-]_i$). With maturation, the effect of these transmitters switches to inhibitory, resulting from a decrease of $[Cl^-]_i$ attributable to a developmental change of $[Cl^-]_i$ regulation, such as an increase of K^+-Cl^- cotransporter (KCC) (Plotkin et al., 1997; Kakazu et al., 1999; Rivera et al., 1999) and a decrease of Na^+-dependent Cl^- transport (Kakazu et al., 1999). In mature neurons, a diversity of KCC function is closely linked to maintenance of GABAergic inhibitory synaptic potentials during repetitive stimulation (Ueno et al., 2002). In various pathophysiological conditions, GABA has been reported to alter neuronal excitation. In cultured hypothalamic neurons injured by scraping, GABA evoked an increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) (van den Pol et al., 1996). In brain slice preparation, tetanic stimulation switched GABA response to excitation as a result of an increase in $[K^+]_o$, which probably disturbs Cl^- extrusion (Taira et al., 1997). Furosemide-sensitive mechanisms are also upregulated in global ischemia (Reid et al., 2000).

In the present study, we focused on elucidating the alterations

Received Jan. 14, 2002; revised March 15, 2002; accepted March 21, 2002.

This work was supported by Grants-in-Aid for Scientific Research on Priority Areas (C)–Advanced Brain Project Grant 13210108, Priority Areas (A)–Integrated Brain Research Grant 13035036, and Priority Areas (A)–Neuronal Circuit Grant 12053256 from the Ministry of Education, Culture, Sports, Science, and Technology, Japan (J.N.). We give our appreciation to Drs. M. Andressen at the Oregon Health Science University and R. Balice-Gordon at the University of Pennsylvania for critical reading of this manuscript.

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of $[Cl^-]_i$ and GABA function and its underlying mechanisms in vagal motoneurons receiving *in vivo* axotomy.

MATERIALS AND METHODS

Surgical procedures. All experiments conformed to the Guiding Principles for the Care and Use of Animal approved by the Council of the Physiological Society of Japan, and all efforts were made to minimize the number of animals used and their suffering.

Unilateral cervical vagus was cut in 16- to 18-d-old rats ($n = 28$) deeply anesthetized with diethyl ether as described previously (Furukawa et al., 2000). Axotomy of vagal motoneurons was made with fine scissors by cutting unilaterally the vagal bundle at the neck along the vagus nerve. For experiments using acutely dissociated neurons of the dorsal motor nucleus of the vagus (DMV neurons), a piece of DiI or rhodamine-conjugated dextran was placed on proximal cut site of the nerve bundle to identify axotomized DMV neurons (Nabekura et al., 1995). The skin incision was repaired, and rats were returned to the cage after awakening from the anesthetic.

Preparation of DMV neurons. DMV neurons were acutely dissociated 1–3 d after *in vivo* axotomy as described previously (Furukawa et al., 2000). Briefly, rats were deeply anesthetized with pentobarbital sodium (50 mg/kg, i.p.), and coronal sections of brainstem (400-μm-thick) were made with a microslicer (VT-1000; Leica, Nussloch, Germany). Thereafter, DMV neurons were mechanically dissociated with fire-polished glass pipettes in a small plastic culture dish (Nabekura et al., 1996). The neurons receiving axonal injury were identified as DiI or rhodamine positive under epifluorescence microscope (Nabekura et al., 1995) (see Fig. 2*A*).

Solutions. Incubation extracellular solution contained (in mM): 124 NaCl, 5 KCl, 1.2 KH_2PO_4, 1.3 $MgSO_4$, 2.4 $CaCl_2$, 10 glucose, and 24 $NaHCO_3$ (well oxygenated with 95% O_2–5% CO_2 gas mixture). The pH was 7.4. Incubation solution was used as extracellular solution for slices before cell dissociation and also used as perfusate in some recordings as indicated. Standard external solution contained (in mM): 150 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, and 10 glucose. The pH was adjusted to 7.4 with Tris base. The patch pipette solution for gramicidin perforated-patch recording contained (in mM): 150 KCl and 10 HEPES. Pipette solutions were buffered to pH 7.2 with Tris-OH. Gramicidin was first dissolved in methanol to prepare a stock solution of 10 mg/ml and then diluted to a final concentration of 100 μg/ml in pipette solutions. Rapid change of external solutions with and without drugs was performed with the "Y-tube" method described previously (Nabekura et al., 1993). Physiological measurements were mainly performed in the standard solution at room temperature (23–26°C), but some were performed at 33°C as stated.

$[Ca^{2+}]_i$ measurement. Coronal brain slices, including the DMV neurons, were loaded with fura-2 in an incubation solution containing 50 μM fura-2 AM with 0.01% cremophore EL (Sigma, St. Louis, MO) at 28°C for 1 hr. After washing fura-2 AM, slices were placed on glass-bottomed recording chamber on the stage of inverted microscope (TMD-300; Nikon, Tokyo, Japan). Ratiometric excitation was provided by a xenon lamp filtered sequentially using microscopic fluorometer (CAM 230; Jasco, Tokyo, Japan).

Electrical measurements. Electrical measurements were performed with gramicidin perforated-patch recording (Kakazu et al., 2000). The resistance between the patch pipette filled with the internal solution and the reference electrode in the normal external solution was 3–5 MΩ. Ionic currents were measured with a patch-clamp amplifier (EPC-7; List Biologic, Campbell, CA). The membrane potential was held at −50 mV throughout the experiment, except as otherwise stated. The voltage ramps (±10–30 mV) consisted of a linear depolarizing voltage command with a frequency of 0.25 Hz (Nabekura et al., 1996). The $E_{GABA\text{-}A}$ was obtained as the membrane potential at which current responses to voltage ramps applied just before and during $GABA_A$ receptor activation intersected each other in each neuron in the presence of 10^{-7} M TTX and 10^{-4} M La^{3+} (Kakazu et al., 1999) (see Fig. 2*B*). To obtain the relationship between resting potentials and resting $E_{GABA\text{-}A}$ in each neuron (see Fig. 2*C,D*), first, resting membrane potential (V_{rest}) was measured and then membrane was clamped at V_{rest}, and voltage ramps were applied in each neuron.

In situ *hybridization.* One to 2 d after receiving ipsilateral vagal axotomy, rats were anesthetized with 50 mg/kg pentobarbital, and brains were quickly removed and frozen using isopentane and stored at −70°C. Ten micrometer sections were cut on a sliding microtome. To detect KCC2 transcripts, two independent methods were used. One used an 189 bp cDNA fragment of rat KCC2 (GenBank accession number U55816; position 197–385) amplified by reverse transcription-PCR from adult rat cerebral RNA (5′ primer, TTCATCAACAGCACGGACAC; 3′ primer, CTTCTTCTTTCCGCCCTCAT). cRNA probes were labeled with digoxigenin (DIG) using standard methods, and *in situ* hybridization was performed. In the second method, a probe for KCC2 mRNA (GenBank accession number U55816) was complementary to bases 2981–3016 (5′-TGGCTTCTCCTCGTTGTCACAAGCTGTCTCTTCGGG-3′) of rat KCC2 mRNA sequence (GenBank). A probe for Na^+, K^+-Cl^- cotransporter (NKCC1) mRNA (GenBank accession number AF051561) complementary to bases 2981–3016 (5′-ACATCCTTGGTACCAGGTGAC-TTTTCTTGTGATGAC-3′) was also used. *In situ* hybridization histochemical technique was described previously (Kanaka et al., 2001). Briefly, probes were labeled at the 3′ end using [α-^{35}S]dATP [1000–1500 Ci/mmol (37–55.5 TBq/mmol); NEN, Boston, MA) and terminal deoxynucleotidyl transferase (TaKaRa, Tokyo, Japan) to obtain a specific activity of ~1.4–2.0 U × 10^9 dpm/ml. Tissue sections were coated with Kodak NBT-2 emulsion (Eastman Kodak, Rochester, NY) diluted 1:1 with water and exposed at 4°C for 2–4 weeks in a tightly sealed dark box. After being developed in D-19 (Eastman Kodak), fixed with photographic fixer, and washed with tap water, the sections were counterstained with thionin solution to allow morphological identification. The number of grains over each neuron was counted.

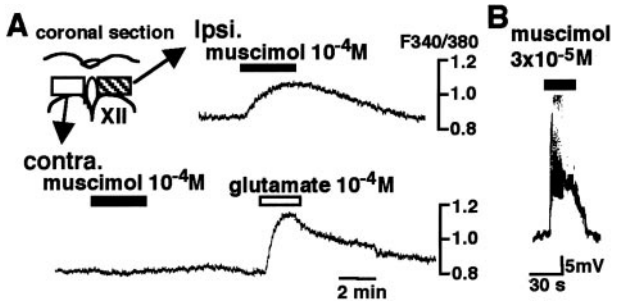

Figure 1. $GABA_A$ receptor mediated-excitation in axotomized DMV neurons. *A*, 10^{-4} M muscimol increased $[Ca^{2+}]_i$ in the DMV neurons ipsilateral (*Ipsi.*) to axotomy (*hatched square*) at 1 d after vagal axotomy. On the other hand, 10^{-4} M glutamate, but not muscimol, increased $[Ca^{2+}]_i$ in the DMV neurons contralateral (*contra.*) to axotomy (*open square*). Both recordings were obtained from same slice. *XII*, Hypoglossal nucleus. *B*, Typical example of muscimol-induced action potentials in an acutely dissociated DMV neuron with axotomy.

RESULTS

Coronal brainstem slices of 400 μm thickness, including the bilateral DMV neurons, were obtained from rats 1–3 d after unilateral vagal axotomy. In slices treated with 50 μM fura-2 AM, application of 10^{-4} M muscimol increased $[Ca^{2+}]_i$ in the DMV neurons ipsilateral to the axotomy in HEPES-buffered standard extracellular solution (nominally HCO_3^- free; $n = 8$) (Fig. 1*A*). This muscimol-induced $[Ca^{2+}]_i$ increase was completely blocked by 10^{-6} M bicuculline ($n = 2$), 10^{-3} M Ni^+ ($n = 4$), and the removal of extracellular Ca^{2+} ($n = 2$). In the presence of 3 × 10^{-7} M TTX, 10^{-3} M muscimol did not increase $[Ca^{2+}]_i$ ($n = 3$). Indeed, muscimol generated action potentials in dissociated axotomized DMV neurons (three of eight cells examined) (Fig. 1*B*). In contrast, muscimol did not increase $[Ca^{2+}]_i$ in the DMV neurons contralateral to axotomy, but glutamate (10^{-4} M) did elevate $[Ca^{2+}]_i$ ($n = 5$). Thus, muscimol-evoked action potentials activated voltage-dependent Ca^{2+} entry and increased $[Ca^{2+}]_i$. Furthermore, when 10^{-4} M muscimol was applied to brainstem slices prepared 6 hr after axotomy, it failed to elevate $[Ca^{2+}]_i$ in the injured DMV neurons ($n = 4$), suggesting that >6 hr are required for GABA to induce neuronal excitation in the DMV neurons after cervical axotomy.

Muscimol-induced membrane depolarization might be attributable to a Cl^- efflux through $GABA_A$ receptor if $[Cl^-]_i$ increases after injury. The direction of Cl^- flux through Cl^- chan-

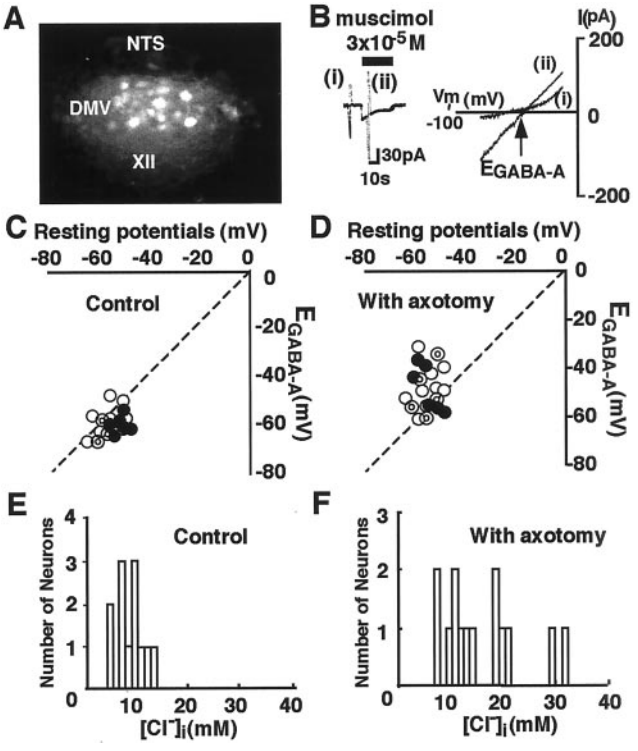

Figure 2. Relationship between resting membrane potential (V_{rest}) and reversal potential of $GABA_A$ receptor response ($E_{GABA\text{-}A}$) in control and axotomized neurons. *A*, Epifluorescent image of injured DMV neurons stained with DiI. *NTS*, Nucleus of tractus solitarius; *XII*, hypoglossal nucleus. *B*, Gramicidin perforated-patch recording was used on the acutely dissociated injured DMV neurons stained with DiI. *B*, *Left*, Voltage ramps were applied before (*i*) and during muscimol application (*ii*). *Right*, Membrane potential at which current responses to voltage ramps intersected with each other (*i*, *ii*) was defined as the $E_{GABA\text{-}A}$. *C*, *D*, $E_{GABA\text{-}A}$ values measured in HEPES-buffered extracellular solution at room temperature (*open circles*, *double circles*) and in HCO_3^--buffered extracellular solution at 33°C (*filled circles*) were plotted as a function of V_{rest} in DMV neurons with ($n = 20$) and without ($n = 18$) axonal injury. *Open* and *double circles* were obtained by using muscimol and GABA applications, respectively. *Filled circles* were obtained by muscimol application. Note the number of neurons with $E_{GABA\text{-}A} > V_{rest}$ increases in the injured group. *E*, *F*, $[Cl^-]_i$ was calculated by using a Nernst equation with knowing $[Cl^-]_o$ and $E_{GABA\text{-}A}$ measured in standard (HEPES-buffered) solution in each neuron.

nel is set by an electrochemical gradient of Cl^-. To better understand Cl^- efflux through Cl^- channels in injured neurons, we first examined the relationship between the resting membrane potentials (V_{rest}) and the reversal potentials for $GABA_A$ receptor-mediated response ($E_{GABA\text{-}A}$) in axotomized DMV neurons.

After acute dissociation of DMV neurons, gramicidin perforated-patch recording, allowing electrical recording with $[Cl^-]_i$ undisturbed, was performed on identified neurons. Current responses to 3×10^{-5} M GABA in control ($n = 4$) and axotomized ($n = 6$) neurons were completely blocked by 10^{-5} M bicuculline, suggesting that the GABA responses observed were mediated by $GABA_A$ receptor activation. In 9 of 14 axotomized DMV neurons tested in standard extracellular solution, $E_{GABA\text{-}A}$ was more depolarized than the resting membrane potential (Fig. 2*D*, *open* and *double circles*). The resting membrane potential and $E_{GABA\text{-}A}$ averaged -53.9 ± 4.9 mV (mean ± SD; $n = 14$) and -46.7 ± 9.8 mV ($n = 14$) in injured DMV neurons compared with -56.2 ± 4.9 mV ($n = 12$) and -60.1 ± 6.2 mV ($n = 12$) in controls, respectively. Thus, $E_{GABA\text{-}A}$ was significantly depolarized in axotomized neurons compared with control DMV neurons ($p < 0.01$; unpaired *t* test), whereas resting membrane potentials were similar ($p > 0.1$). Because $[Cl^-]_o$ is constant in our perfusate, we calculated $[Cl^-]_i$ in each neuron based on the Nernst equation using measured $E_{GABA\text{-}A}$ and the $[Cl^-]_o$. $[Cl^-]_I$ values were calculated to be 17.1. ± 7.3 mM (mean ± SD) in axotomized DMV neurons ($n = 14$) (Fig. 2*F*) and 10.1 ± 2.7 mM in control neurons ($n = 12$) (Fig. 2*E*). A significant difference was observed between the two values ($p < 0.01$; unpaired *t* test). Thus, with elevated $[Cl^-]_i$, an opening of Cl^- channel induces an efflux of Cl^- out of the cell, resulting in membrane depolarization in axotomized DMV neurons.

To confirm more depolarized $E_{GABA\text{-}A}$ in injured neurons under more physiological conditions, we also measured $E_{GABA\text{-}A}$ in the incubation solution (HCO_3^- buffered) at 33°C. $E_{GABA\text{-}A}$ was more depolarized in the injured neurons (51.7 ± 9.2 mV; mean ± SD; $n = 6$) than in the control (61.7 ± 3.7 mV; $n = 6$; $p < 0.05$) (Fig. 2*C*,*D*, *filled circles*), whereas the resting potential was not significantly affected after axotomy ($p > 0.1$). No significant difference in $E_{GABA\text{-}A}$ was observed between HEPES-buffered and HCO_3^--buffered extracellular solutions in the control and axotomized neurons ($p > 0.1$ in each group; unpaired *t* test).

Alteration of cation–Cl^- cotransporters after axonal injury

In central neurons, the regulation of $[Cl^-]_i$ is primarily driven by cation–Cl^- cotransport processes, e.g., KCC and NKCC (Kakazu et al., 1999; Rivera et al., 1999; Ueno et al., 2002). Indeed, 1 mM furosemide, an inhibitor of the transporters, almost completely eliminated the differences in $E_{GABA\text{-}A}$, which were near the holding potential (V_h of − 50 mV) in both control ($n = 6$) and axotomized ($n = 6$) neurons (Fig. 3*A*). This result suggests an active role for the furosemide-sensitive transporters, e.g., KCC and NKCC, in maintaining $[Cl^-]_i$ low in control DMV neurons and high in injured neurons. In addition, reducing the driving force for K^+ by raising $[K^+]_o$ from 5 to 20 mM ($[K^+]_i$, 150 mM) induced a positive shift of $E_{GABA\text{-}A}$ in the control ($n = 3$) (Fig. 3*B*), suggesting that a furosemide- and K^+-sensitive mechanism, e.g., KCC, maintains a low $[Cl^-]_i$ in control neurons.

To examine the functional involvement of NKCC, another furosemide-sensitive Cl^- cotransport, the effect of bumetanide on $[Cl^-]_i$ was examined in control and injured neurons. In the presence of 10 μM bumetanide, $[Cl^-]_i$ decreased by 3.9 ± 1.2 mM ($n = 5$) in the injured neurons and 2.1 ± 0.8 mM ($n = 5$) in the control neurons. Removal of extracellular Na^+ ($n = 4$) with the aim to inhibit Na^+-dependent Cl^- transport, e.g., NKCC, gradually decreased $[Cl^-]_i$ in injured neurons (Fig. 4). Thus, NKCC seems to play an important role in keeping high $[Cl^-]_i$ in injured neurons.

Of the cation-Cl^- transporters, KCC2 (Payne et al., 1996) and NKCC1 (Plotkin et al., 1997) are predominately expressed in the CNS. *In situ* hybridization revealed that mRNA for KCC2 was significantly less prevalent in axotomized DMV compared with the control side at 1–3 d after axotomy (Fig. 5). Reduction in KCC2 mRNA expression was also observed in the hypoglossal ($n = 5$) and facial nuclei ($n = 3$) ipsilateral to axotomy performed at XII and VII cranial nerves, respectively. Thus, reduction of KCC2 mRNA after axotomy was not restricted to the DMV neurons, but may be a common phenomenon in the motor neurons. In addition, "crush" injury of vagal bundle at the neck mimicked cut injury regarding reduced KCC2 mRNA expression in DMV neurons ($n = 6$). On the other hand, the density of NKCC1 mRNA did not change between the DMV neurons

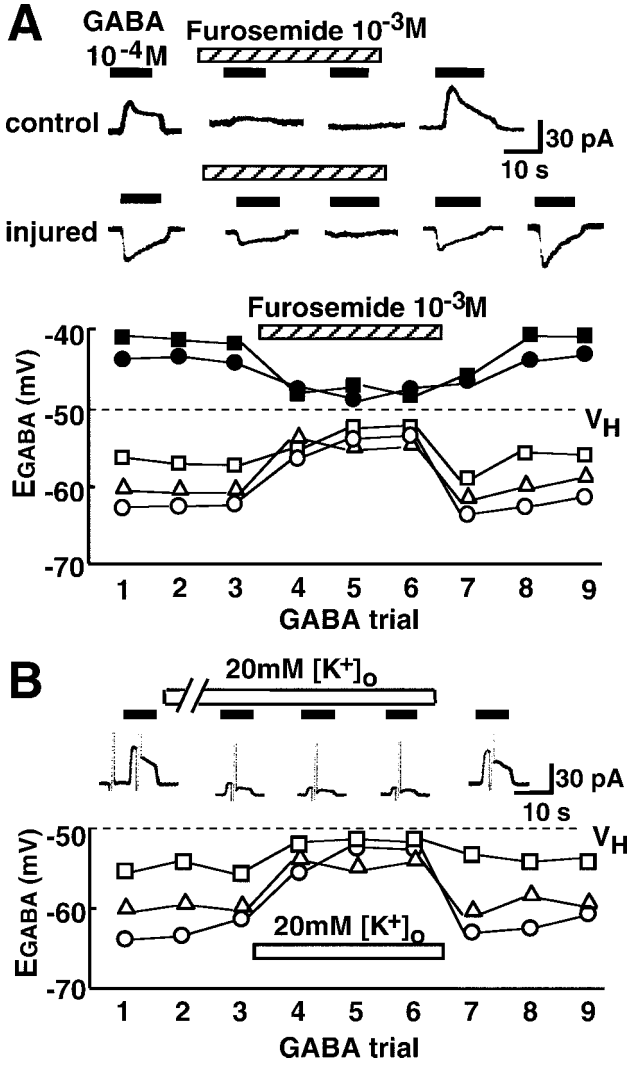

Figure 3. Furosemide-sensitive and K^+-dependent $[Cl^-]_i$ regulation. *A*, Furosemide at 1 mM reversibly decreased the amplitudes of GABA responses (*current traces*) and shifted E_{GABA-A} to a V_h in the DMV neurons with (*filled circles*) and without (*open circles*) axotomy (*bottom graphs*). Thus, E_{GABA-A} values in both groups were maintained mainly by furosemide-sensitive mechanisms. Representative three of six control and two of six injured neurons were shown in the graph. *B*, An increase of $[K^+]_o$ from 5 to 20 mM to decrease K^+-driving force across the membrane decreased the outward amplitude of GABA response (*top traces*) and depolarized E_{GABA-A} in the control neurons (*bottom graph*). *Vertical lines* in the current traces were current responses to ramp voltage commands applied. $[K^+]_i$, 150 mM. V_h was −50mV. GABA (10^{-5} M) was applied at an interval of 15 min.

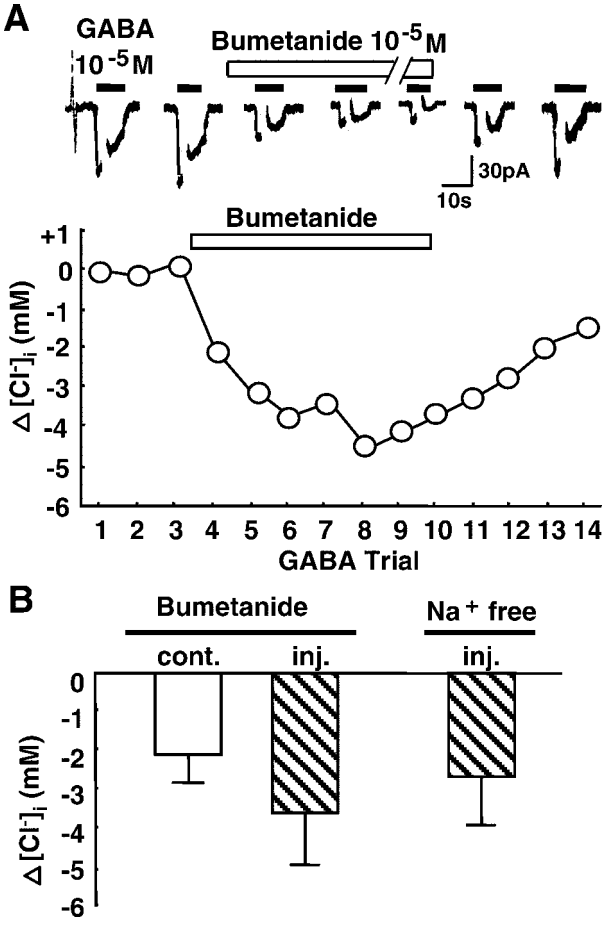

Figure 4. Cl^- accumulation by bumetanide- and Na^+-sensitive mechanism in injured neurons. *A*, *Top traces*, In the presence of 10^{-5} M bumetanide, the amplitude of inward current response to 10^{-5} M GABA gradually decreased in injured neurons. After the end of bumetanide, inward GABA response gradually restored (*top current traces*). Disruption of current trace during each GABA application is attributable to rapid current response to voltage ramp to measure the E_{GABA-A}. GABA was applied at an interval of 10 min. V_h was −50mV. *Bottom graph*, In this neuron, a reversible disturbance of Cl^- accumulation by 10^{-5} M bumetanide was demonstrated. Axotomy was performed 2 d before making preparation. *B*, Decreases of $[Cl^-]_i$ resulted from disturbance of Cl^- accumulation by 10^{-5} M bumetanide in the control (*cont.; n* = 5) and injured (*inj.; n* = 5) neurons and by a removal of extracellular Na^+ in injured neurons (*n* = 4).

ipsilateral and contralateral to axotomy (Fig. 6). Thus, the increase of Cl^- over the holding potential or resting potential is attributable to a decrease of Cl^- extrusion by KCC and to a lesser affected Cl^- accumulation mechanism attributable to NKCC, resulting in excess Cl^- accumulation.

DISCUSSION

GABA-induced excitation in injured neurons

In the present study, an increase of $[Ca^{2+}]_i$ evoked by $GABA_A$ receptor activation appeared after 6 hr after *in vivo* axonal injury. In cultured neurons, a rapid alteration of $[Cl^-]_i$ with a latency of <3 hr after scraping injury applied directly to the soma has been reported previously (van den Pol et al., 1996). Increases of $[Ca^{2+}]_i$ induced by $GABA_A$ receptor activation after injury are blocked by Ni^{2+} in both preparations, suggesting that GABA-induced $[Ca^{2+}]_i$ increase is mediated by Ca^{2+} channel activation in the plasma membrane of neurons with *in vivo* and *in vitro* injuries.

Difference in distance between the site of injury and soma might contribute to the discrepancy in the onset of GABA excitation after neuronal injury. In *Aplysia*, the axonal "injury signals" move to the soma at a rate of 36 mm/d (Gunstream et al., 1995). Slow conduction of an injury signal from the injured site to the soma might account for the delayed onset in the present experiment. Alternatively, soma scraping might be a more potent injury than axonal injury regarding alterations in Cl^- regulation.

GABA-elevated $[Ca^{2+}]_i$ in the DMV neurons ipsilateral to axotomy was mediated by voltage-dependent Na^+ channel activation because TTX blocked GABA-induced neuronal excitation. Thus, GABA-induced depolarization possibly elicits Na^+ channel activation and further membrane depolarization, resulting in

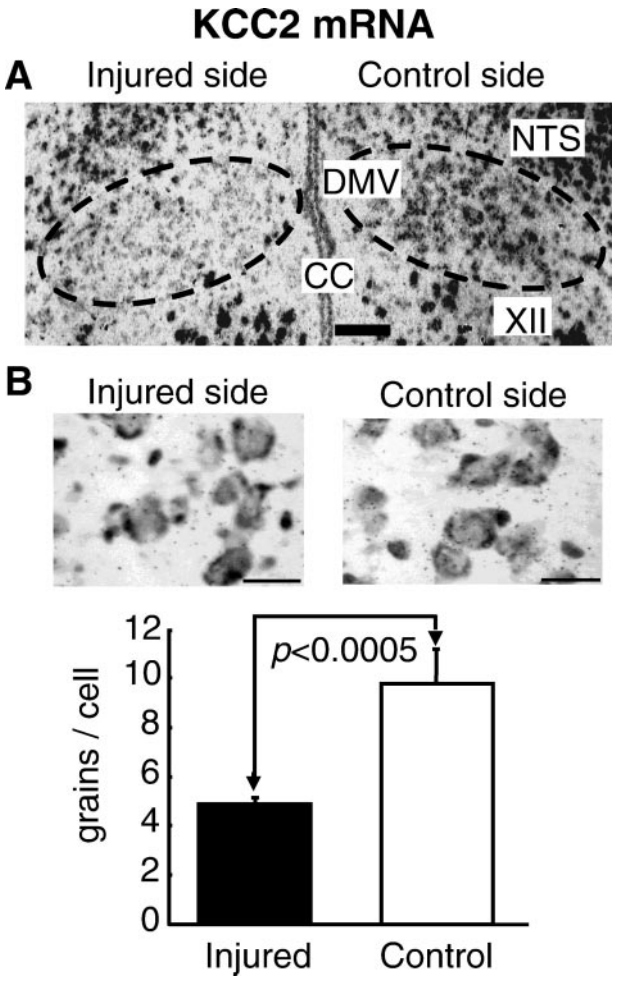

Figure 5. *In situ* hybridization of KCC2 mRNA in the DMV neurons with and without axotomy by using DIG-labeled probe (*A*) and ^{35}S-labeled probe (*B*). Cut injury of X nerve at the neck was performed 2 d before making preparation. *A*, *In situ* hybridization by using DIG-labeled probe revealed less density of KCC2 mRNA expressions in the DMV neurons ipsilateral to axotomy (*Injured side*) than that contralateral to axotomy (*Control side*). DMV neurons are surrounded by *dotted lines*. *XII*, Hypoglossal nucleus; *NTS*, nucleus tractus solitarius; *CC*, central canal. *B*, KCC2 mRNA hybridization signals by using ^{35}S-labeled probe (*small black dots*) were severely downregulated in injured DMV neurons. The sections were counterstained with thionin solution to allow morphological identification of cells (*gray areas*). *Bottom graph*, Comparisons of the number of grains in the DMV neuron expressing KCC2 mRNA. All data are means ± SD ($n = 4$). Statistics were performed with Student's *t* test. Marked decrease in the numbers of grains in the DVM neuron with axotomy. Scale bars: *A*, 80 μm; *B*, 20 μm.

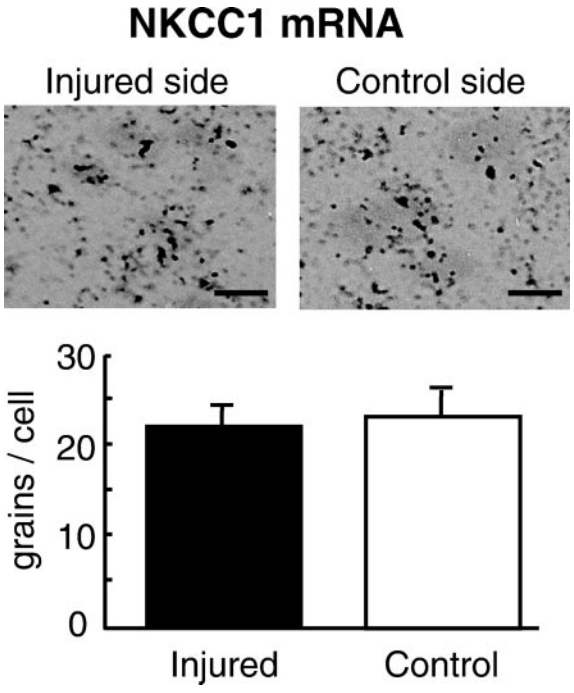

Figure 6. *In situ* hybridization of NKCC1 mRNA in the DMV neurons with and without axotomy. *Top*, Bright-field photomicrographs of a counterstained section shows that no remarkable difference of NKCC1 mRNA (*black dots*) was observed between the DMV neurons with (*Injured side*) and without (*Control side*) axotomy. Scale bar, 20 μm. *Bottom*, Comparisons of the number of grains in the DMV neuron expressing NKCC1 mRNA. All data are means ± SD ($n = 4$). Statistics were performed with Student's *t* test. No significant difference was obtained between two groups ($p > 0.1$).

activation of voltage-dependent Ca^{2+} channel. Muscimol induced membrane depolarization in a majority of injured motoneurons (Fig. 2*D*) and was also able to elicit action potentials in injured neurons (Fig. 1*B*).

Alteration of intracellular Cl^- regulators

Activation of $GABA_A$ receptors allows Cl^- movement mainly according to the difference between E_{Cl-} and membrane potential. In the present result, no significant difference in the resting membrane potential was obtained between control and injured neurons. Thus, alteration of GABA action is attributable to a change of $[Cl^-]_i$ because of the constant $[Cl^-]_o$ in our experimental condition. Increase of $[Cl^-]_i$ over the V_h and resting potential is caused by net $[Cl^-]_i$ accumulation with or without a decrease of Cl^- extrusion in injured neurons. At present, various $[Cl^-]_i$ regulators, such as KCC, NKCC, Cl^--HCO_3^- exchange, and Na^+-dependent Cl^--$HCO3^-$ exchange, are known to be involved in the regulation of $[Cl^-]_i$ in the central neurons (Kaila, 1994). Because our solution is HEPES based, changes of Cl^-–HCO_3^- exchange and Na^+-dependent Cl^-–HCO_3^- exchange are not required for regulating $[Cl^-]_i$ during our responses. Of these regulators, KCC, which normally carries Cl^- out of the cell with K^+, primarily contributes to keep low $[Cl^-]_i$ in the mature neurons (Fig. 3) (Kakazu et al., 2000; Ueno et al., 2002). Indeed, developmental increase of KCC2 mRNA primarily contributes to switch GABA response from the excitation to the inhibition in the hippocampal neurons (Rivera et al., 1999). Neuronal diversity in KCC2 expression is closely linked to that of Cl^- extrusion efficacy (Ueno et al., 2002). On the other hand, NKCC carries Cl^- into the cell by using cationic driving forces, which helps to keep a high $[Cl^-]_i$ in immature neurons (Plotkin et al., 1997; Kakazu et al., 1999). In the DMV neurons, furosemide- and cation-sensitive mechanisms contributed substantially to maintain $[Cl^-]_i$ constant in control and injured DMV neurons (Figs. 3, 4).

Functional alteration of furosemide-sensitive cation-Cl^- cotransporters in the injured neurons could be accounted for by changes in either (1) activity of existing KCC and NKCC or (2) KCC and NKCC expression. In several non-neuronal cell types, NKCC and KCC activities are modulated by phosphorylation–dephosphorylation mechanisms (Krarup et al., 1998; Jennings 1999; Lauf and Adragna, 2000; Russell, 2000) by cAMP-dependent processes (Greger et al., 1999) and by myosin chain kinase (Kelley et al., 2000). Thus, it cannot be ruled out that

changes in various intracellular substances, such as protein kinases, after axotomy might affect existing cation–Cl^- cotransporter activities. Our *in situ* hybridization studies demonstrated a decrease in KCC2 mRNA (Fig. 5), whereas NKCC1 mRNA was unchanged in the axotomized DMV neurons (Fig. 6). A significant role of NKCC1 in keeping high $[Cl^-]_i$ has been clearly demonstrated in sensory afferent neurons (Sung et al., 2000). In our hands, 10 μM bumetanide, a potent NKCC inhibitor, as well as removal of extracellular Na^+ decreased $[Cl^-]_i$ in injured neurons, as evidenced by GABA-induced inward currents recorded in our standard extracellular solution (Fig. 4). Thus, we conclude that, in injured neurons, the Cl^- accumulation is attributable to a decrease in Cl^- extrusion, which is mediated by reduced KCC2 expression, whereas influx of Cl^- through NKCC1 remained unchanged. As a consequence, this is likely to be responsible for switching GABA action to excitation.

Functional significance of injury-induced alteration of GABA responses

Reappearance of immature characteristics after *in vivo* axonal injury has been observed for $\alpha 3$, $\beta 4$, and $\alpha 5$ ACh receptor subunits in the ciliary ganglion (Levey and Jacob, 1996) and type III Na^+ channels in dorsal root ganglion neurons (Waxman et al., 1994). As for $[Cl^-]_i$ regulation, lack of KCC and maintenance of NKCC function in the lateral superior olive neurons (Kakazu et al., 1999) and less KCC2 mRNA expression in the hippocampus (Rivera et al., 1999) have been demonstrated in immature animals. In DMV neurons, immature characteristics of GABA responses (present results), as well as NMDA responses (Furukawa et al., 2000), appear soon after axonal injury. GABA enhances neurite outgrowth and induces synaptic maturation in immature animals (Spoerri, 1988; Barbin et al., 1993). Thus, in this sense, neuronal injury might result in neurons reacquiring more plastic characteristics. On the contrary, it is possible that GABA-induced depolarization in injured neurons activates voltage-dependent Ca^{2+} channels and relieves the voltage-dependent Mg^{2+} block of NMDA receptors (Furukawa et al., 2000). An increase of intracellular Ca^{2+} via these mechanisms might be related to neuronal Ca^{2+}-dependent excitotoxicity, such as phospholipases, endonucleases (Choi, 1992), and calpain (Siman et al., 1989).

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